DOCKET NO.: 790076.401

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF : Michael Giesing et al.

SERIAL NO. : 09/485,879

FILED : June 22, 2000

FOR: Method for the characterization of disseminated and micrometastasized cancer cells

DECLARATION UNDER 37 C.F.R. §1.132

COMMISSIONER OF PATENTS
P.O. BOX 1450
ALEXANDRIA, VA 22313-1450

SIR:

Now comes Prof. Dr. med. Michael Giesing who deposes and states:

1. I am a graduate of University of Bonn, Germany, and received my doctorate degree in the year 1971.

2. I have been working for 14 years as a laboratory physician in the field of molecular oncology.

3. I have read and fully understood U.S. application, Ser. No. 09/485,879.

4. I have read and fully understood the Office Action of September 20, 2004 and the prior art cited therein.

5. The following experiments and investigations were carried out by me or under my direct supervision.

A number of patients suspected of having cancer were examined.

From each patient peripheral blood was collected in heperanized Vacotainer systems (Beckton Dickensen). Mononucleated cells (MNC) from 12.2 ml peripheral blood were

purified over a density gradient using Nycoprep 1.077 (Nycomed, Norway). Collected MNC fractions were washed twice with PBS (0.2 % BSA; Life Technologies, Germany) and resuspended in 12.2 ml PBS (0,2 % BSA).

Cells derived from 5 ml of the MNC suspension were lysed in Trizol® (Life Technologies, Germany) and stored (fraction A).

7 ml of the MNC suspension were used for subsequent isolation of disseminated cancer cells by size using a column containing a polyester mesh (mesh-opening 20 µm; RELAB AG, Germany). The MNC suspension was pipetted onto the mesh. After the MNC suspension had completely passed the mesh, this was rinsed 10 times using 5 ml PBS each. The cells retained on the mesh were lysed using Trizol® (Life Technologies, Germany) and stored (fraction C).

RNA from fractions A and C was extracted using commercially available RNA purification kits according to the manufacturers instructions (Qiagen, Germany).

The purified RNA was then assessed for mRNAs encoding the following proteins:

A1) Prostate-specific membrane antigen (PSM).

A2) Prostate-specific antigen (PSA).

A3) Androgene receptor (AR).

B1) Manganese superoxide dismutase (SOD).

B2) Thioredoxin reductase 1 (TXNRD1).

B3) Glutathione peroxidase 1 (GPX1).

The experimental protocols for assessing the mRNAs A1), A2) and A3) are described in Exhibit A attached hereto and the experimental protocols for assessing the mRNAs B1), B2) and B3) are described in Exhibit B attached hereto.

PSM-, PSA-, and AR-mRNA (A1, A2, A3) are essentially not expressed in blood borne non-cancer cell. Consequently, these mRNAs are prostate-specific and therefore qualify as organotypical nucleic acids. Detection of such mRNA in blood therefore indicates the presence of prostate-derived cells. If said prostate-derived cells were disseminated cancer cells, the presence of such mRNA would also indicate the type of malignant disease from which the disseminated cancer cells are derived.

The assessment of said prostate-specific mRNAs was carried out with fraction A, which comprises mainly mononuclear cells and, if present at all, a comparatively small proportion of prostate-derived cells which may qualify as cancer cells. Since said mRNAs are essentially not expressed in non-cancer cells, i.e. the mononuclear cells, the presence of at least one of said prostate-specific mRNAs in fraction A was considered as an indication that the patient examined had prostate-derived cells in blood (positive for prostate-derived cells).

In contrast to said prostate-specific mRNAs, the SOD-, TXNRD1-, and GPX1-mRNAs are, to a certain extent, also expressed in blood borne non-cancer cells, i.e. the mononuclear cells. An enhanced expression of said mRNAs, however, is considered to be associated with cancer.

The assessment of these cancer-associated mRNAs was carried out with both fraction C (the cancer cell fraction) and fraction A (the control cell fraction comprising mainly non-cancer cells such as mononuclear cells). An enhanced expression of at least one of said

mRNAs in fraction C (the cancer cell fraction) relative to the expression of said mRNAs in fraction A (the mononuclear cell fraction) was considered as indicating an increased risk of the patient concerned for having disseminated cancer cells in blood (positive for disseminated cancer cells).

Among the patients examined, there were 30 patients assessed positive for having prostate-derived cancer cells (based on the presence of at least one mRNA selected from A1, A2 and A3). From these 30 patients, however, only 11 patients were also positive for disseminated cancer cells (based on the enhanced expression of at least one mRNA selected from B1, B2 and B3). The malignant nature of these cells has been also confirmed by the detection of aberrant DNA, i.e. oncogenes and allelic losses of tumor suppressor genes.

The clinical follow-up of said 30 patients revealed that 100 % patients which were assessed positve for both prostate-derived cancer cells and disseminated cancer cells had indeed a prostate tumor. In the remaining patients (positive for prostate-derived cancer cells but negative for disseminated cancer cells), however, a tumor could not be found. These patients may have suffered from prostatitis or benign prostate hypertrophy. In some cases prostate-derived cells may have been released into the blood stream by preceding rectal examination or biopsy.

Surprisingly, further investigations have revealed that the prostate-specific mRNAs PSM and PSA could not be detected in fraction C (the cancer cell fraction), even if they were present in fraction A obtained from the very same blood sample of a given patient. For AR-mRNA, in the majority of cases this mRNA could not be detected in fraction C, even though it was present in fraction A.

While the above relates to prostate cancer, I have made the same experience with other types of cancer, in particular breast cancer.

The above clearly shows that, with a method for characterizing disseminated cancer cells in a body fluid such as blood, the advantage of not only reliably detecting disseminated cancer cells but also providing further cancer-related information, e.g. about the source of spread, is achieved only if both, at least one mRNA that is essentially not expressed in non-cancer cells in the body fluid and at least one further cancer-related nucleic acid in a cancer cell fraction versus a non-cancer cell fraction are investigated. With either investigation alone this would not be possible.

7. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

8. Further deponent saith not.

Lienen, Germany, März 11-05 .....

Attachments:

Exhibits A, B

Exhibit A

Detection of PSM-mRNA, PSA-mRNA, AR-mRNA

The amounts of expressed prostate-specific membrane antigen (PSM)-mRNA, prostate-specific antigen (PSA)-mRNA and androgene receptor (AR)-mRNA were determined by quantitative RT-PCR. The PCR format was based on the 5'-exonuclease assay known per se (TaqMan®) and was suitable for use on the TaqMan® 7700 sequence detector from Applied Biosystems (ABI).

More specifically, a RT-mix containing first strand buffer, dithiothreitol, RNA guard, random hexamers, dNTPs and reverse transcriptase was prepared. Then, the sample RNA was denatured and immediately cooled on ice, mixed free of air bubbles with the RT-mix, incubated to allow reverse transcription, incubated at higher temperature to stop reverse transcription, immediately cooled on ice and the resulting cDNA either subjected directly to PCR or frozen at –20 °C.

A PCR reaction mixture containing TaqMan® buffer, $MgCl_2$, dNTPs, specific sense primer, specific antisense primer, specific TaqMan® probe and heatstable polymerase was prepared. To this PCR reaction mixture was added the cDNA obtained by reverse transcription and the resulting mixture was subjected to PCR carried out in the ABI 7700 sequence detector (TaqMan®).

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to the relevant mRNA was present in the sample (fraction A). For safety reasons a cutt-off determination only for AR-mRNA detection (AR/GAPDH = 0.1) was additionally chosen in order to exclude even very small amounts of non-prostate derived expression.

Exhibit B

Detection of SOD-mRNA; TXNRD1-mRNA; GPX1-mRNA

The amounts of expressed manganese superoxide dismutase (SOD)-, thioredoxin reductase 1 (TXNRD1)- or glutathione peroxidase 1 (GPX1)-mRNA were determined by quantitative RT-PCR. The PCR format was based on the 5'-exonuclease assay known per se (TaqMan®) and was suitable for use on the TaqMan® 7700 sequence detector from Applied Biosystems (ABI).

More specifically, a RT-mix containing first strand buffer, dithiothreitol, RNA guard, random hexamers, dNTPs and reverse transcriptase was prepared. Then, the sample RNA was denatured and immediately cooled on ice, mixed free of air bubbles with the RT-mix, incubated to allow reverse transcription, incubated at higher temperature to stop reverse transcription, immediately cooled on ice and the resulting cDNA either subjected directly to PCR or frozen at –20 °C.

A PCR reaction mixture containing TaqMan® buffer, $MgCl_2$, dNTPs, specific sense primer, specific antisense primer, specific TaqMan® probe and heatstable polymerase was prepared. To this PCR reaction mixture was added the cDNA obtained by reverse transcription and the resulting mixture was subjected to PCR carried out in the ABI 7700 sequence detector (TaqMan®). A corresponding PCR was carried out using GAPDH-specific sense primer, GAPDH-specific antisense primer and GAPDH-specific TaqMan® probe.

The following SOD-specific primers and probes were used (MNSOD, SOD2; accession No.: M36693):

sense: 5'-GTCACCGAGGAGAAGTACCAGG -3'

antisense: 5'-GGGCTGAGGTTTGTCCAGAA-3'

probe: 5'-CGTTGGCCAAGGGAGATGTTACAGCCC-3'

Size of the PCR product: 131 bp.

The following TXNRD1-specific primers and probes were used (TXNRD1; accession No.: X91247 cDNA):

sense: 5'-GGAGGGCAGACTTCAAAAGCTAC-3'

antisense: 5'-ACAAAGTCCAGGACCATCACCT-3'

probe: 5'-TTGGGCTGCCTCCTTAGCAGCTGCCA-3'

Size of the PCR product: 158 bp.

The following GPX1-specific primers and probes were used (GPX1; accession No.: M21304):

sense: 5'-CTCGGCTTCCCGTGCAA-3'

antisense: 5'-TGAAGTTGGGCTCGAACCC-3'

probe: 5'-AGTTTGGGCATCAGGAGAACGCCAAGAA-3'

Size of the PCR product: 109 bp.

The following GAPDH-specific primers and probes were used (GAPDH; accession No. X01677):

sense: 5'-TGCTGATGCCCCCATGTTC-3'

antisense: 5'-GGCAGTGATGGCATGGACTG-3'

probe: 5'-TCAAGATCATCAGCAATGCCTCCTGCA-3'

Size of the PCR product: 174 bp.

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to SOD-mRNA was present in the sample.

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to the relevant mRNA was present in the sample.

For the evaluation, the ratio of cell equivalents of the relevant mRNA to cell equivalents of GAPDH-mRNA was found for the cancer cell fraction and for the control cell fraction, and the ratio of the resulting quotients was found in turn. A ratio (cancer cells/control cells) significantly higher than 1 was considered as a positive result representing an overexpression of the relevant mRNA in the cancer cell fraction.